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Concentrates of triterpenic acids obtained from crude olive pomace oils: characterization and evaluation of their potential antioxidant activity

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Abstract

BACKGROUND

Pentacyclic triterpenic acids (TA) are phytochemicals of increasing nutritional interest due to their bioactive properties, such as anti-inflammatory, anti-tumoral, anti-hyperglycemic, hepatoprotective and others. Crude olive pomace oils constitute a non-exploited significant source of these compounds. In the present study, concentrates of TA were extracted and characterized from crude olive pomace oils that were obtained by centrifugation and subsequent solvent extraction, respectively. Specifically, the concentrates were obtained from the byproduct generated in the filtration of the oils. The solids were subjected to Soxhlet extractions with hexane to remove the residual oil and then with ethanol for the TA extraction.

RESULTS

Concentrates containing 850-980 g kg^{-1} TA were isolated from the oils obtained by centrifugation, whereas those isolated from oils obtained by hexane extraction presented levels of TA that ranged from 510 to 900 g kg^{-1}. Oleanolic (OA) and maslinic (MA) acids were the TA found in the concentrates. The relative contents of OA and MA were, respectively, 30:70 (p/p) and 77:23 (p/p). All concentrates also presented phenolic compounds at levels of g kg^{-1} and displayed slight antioxidant properties.

CONCLUSION

Concentrates of TA, containing MA and OA, can be readily obtained from a byproduct generated by filtration of crude olive pomace oils. Concentrates isolated from oils obtained by centrifugation were rich in MA, whereas those from oils extracted with hexane were rich in OA. The concentrates showed slight antioxidant properties that can be mainly attributed to the presence of phenolic compounds and not to TA.

Key-words: Triterpenic acids; oleanolic; maslinic; olive pomace oil, by-product reutilization

INTRODUCTION

The industrial production of olive oil in Spain is commonly carried out by applying a two-phase centrifugation system that, apart from the oil, generates a semisolid byproduct called two-phase pomace or "alperujo". In this byproduct, there is still a considerable amount of residual oil that is normally extracted by solvent extraction with hexane. The generation of "alperujo" is so concentrated in time that this has to be stored in large ponds, in which it remains in the open air for months, and the oil is extracted periodically. Before storage, part of the residual oil is separated by a three-phase centrifugation system. The stored "alperujo" is dried by a drastic process and the remaining oil is extracted with hexane. As a result, two crude olive pomace oils are obtained by centrifugation and subsequent solvent extraction from "alperujo".[1,2] In practice, the two oils are mixed and the blend is refined, though they are more and more often used separately.

Apart from the residual oil, "alperujo" also contains significant amounts of valuable bioactive compounds that pass in part to the oil extracted.[3] Such is the case of triterpenic acids (TA), a group of pentacyclic compounds of increasing nutritional interest. Numerous beneficial health properties have been attributed to TA, such as anti-inflammatory, anti-tumoral, anti-hyperglycemic, hepatoprotective, cardioprotective and antimicrobial effects.[4-7] Oleanolic and maslinic acids are the main TA found in olive oils and olive pomace oils.[2] While minor amounts of these compounds are detected in olive oils, significant concentrations occur in olive pomace oils, and especially in crude oils, in which are mostly present as solid particles in suspension because of their very low solubility. Concentrations of almost 8000 mg kg^{-1} of maslinic and oleanolic acid, respectively, have been detected in crude pomace oils obtained by centrifugation,[2] whereas the concentration of TA in virgin olive oils has been reported to be lower than 100 mg kg^{-1} oil.[8] Therefore, crude pomace oils represent a good source of these valuable compounds.

Along with the free fatty acids, TA and other phytochemicals are substantially removed in the conventional refining process with caustic soda.[9] In this regard, our research group has patented a technological approach to increase the levels of TA in edible olive pomace oil obtained by centrifugation.[10] Relatively high amounts of TA are retained in the oil by applying a specific physical refining in which the free fatty acids are removed by successive distillation steps. Previous to refining, the crude oil is filtered leaving a byproduct that can be utilized as raw material to obtain concentrates of TA. Obtaining these concentrates is also the objective of the mentioned patent. The byproduct generated in the filtration step is subjected to a soxhlet extraction with hexane to remove the remaining oil and, subsequently, to a second soxhlet extraction with ethanol to obtain concentrates of TA.

Some of the positive effects of TA against certain diseases have been attributed in part to their antioxidant properties. TA have shown inhibition of lipid peroxidation in rat liver microsomes,[11-13] protection against oxidative damage in DNA[14] and in low-density lipoproteins (LDL),[12,15] and inhibition of cyclo-oxygenase and 5-lipoxygenase activities.[16] Despite the claimed antioxidant properties, a few studies have shown contradictory results. Oleanolic acid did not show free radical scavenging activity by the oxygen radical absorbance capacity (ORAC) assay, whereas maslinic acid acted as an efficient peroxyl radical scavenger.[14,15] Castellano *et al.*[17] found a low activity of oleanolic acid in scavenging the ABTS radical, i.e. 2,2'-azinobis(3ethylbenzothiazoline-6-sulfonic acid), no activity in capturing the DPPH radical, i.e. 2,2-diphenyl-1-picrylhydrazyl, and very low activity in protecting fluorescein in the ORAC assay. They even found that oleanolic acid not only did not prevent oxidation of olive oil at Rancimat conditions but otherwise showed a pro-oxidant effect. The pro-oxidant effect was also evident in refined sunflower oil and this was much higher than that found in the olive oil.

The aim of this study was obtaining and characterizing concentrates of TA from crude olive pomace oils obtained by centrifugation and subsequent solvent extraction, respectively. To the best of our knowledge, concentrates from oils obtained by solvent extraction have not been undertaken so far. Furthermore, due to the controversy concerning the antioxidant activity of TA, the antioxidant activity of the concentrates was explored. For this purpose, the free radical scavenging activities to DPPH and ABTS, to peroxyl radicals in the ORAC assay and the ferric reducing/antioxidant power (FRAP) were studied.

MATERIALS AND METHODS

Chemicals

Oleanolic acid (≥ 97%), maslinic acid (≥ 98%), Trolox (97%), *tert*-butylhydroxytoluene (BHT) (≥ 99%), (±)-α-tocopherol (≥ 96%), DPPH, ABTS, 2,4,6-tris(2-pyridyl)-*s*-tirazine (TPTZ) (≥ 99%), ferric chloride hexahydrate, fluorescein and 2,2-azobis(methylpropionamidine)dihydro (APPH) were acquired from Sigma-Aldrich (Madrid, Spain). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), used as silylation reagent, was purchased from Supelco (Bellefonte, PA, USA).

Oil samples and treatments

Twelve crude olive pomace oils obtained by centrifugation or by subsequent solvent extraction were supplied in 1-L bottles by "Oleícola El Tejar" (Córdoba, Spain). The oils were produced in two consecutive seasons. Specifically, the oils were obtained in January, April and May 2013 (2012/2013 season), and in December 2013, March and June 2014 (2013/2014 season). While the oils of the second season were used immediately as they were received, the oils of the first season had been stored for one year at 5 ºC when they were filtered. About 800 g oil were filtered in the lab using a filter paper. The filtration was carried

out at room temperature. Previously, the oils were homogenized by shaking manually their corresponding bottles. The filtration process lasted about 3 days. The content of the filter paper was subjected to soxhlet extraction. First, hexane was used to remove the oil and, second, ethanol to extract TA. The soxhlet comprised a 1-kg solid container and a 5-L liquid flask. The extraction processes with hexane and ethanol were performed during 4 and 8 h, respectively. The solvent of the ethanolic extract was evaporated in a rotary evaporator at 70 °C and finally in a vacuum oven at 70 °C and 50 mbar until a concentrate in dry powder form was obtained at constant weight. The concentrates obtained from oils produced by centrifugation were named C samples, whereas those from oils extracted with solvent were referred to as S samples. All samples were stored in a refrigerator at 5 °C until analysis.

Analysis of triterpenic acids

The concentrations of TA in the concentrates were analyzed by HPLC according to García *et al.*[2] A volume of 20 μL of a solution of the concentrates in ethanol at 0.5 mg/mL was analyzed directly in a chromatograph equipped with a Waters 717 plus autosampler, a Waters 600 pump, a Waters column heater module and a Waters 996 photodiode array detector whose data were recorded and processed in Empower.2 software (Waters, Inc., Milford, MA, USA). A Spherisob ODS-2 column (Waters, Inc.) (25 cm × 4.6 mm i.d., 5 μm particle size) was used. The separations were performed at 35 °C in isocratic regime using methanol:acidified water with phosphoric acid (pH 3.0) (92:8, v/v) at a flow rate of 0.8 mL min^{-1}. TA were detected at 210 nm and quantified using external calibration.

HPSEC analysis

The concentrate samples were dissolved in tetrahydrofuran at 20 mg mL^{-1} and filtered through a 0.22 μm pore size PTFE filter. While the C samples were completely dissolved in tetrahydrofuran, the S samples presented fines in suspension. A volume of 10 μL of the filtered solutions were analyzed in an HPSEC chromatograph equipped with an AT 1100 autosampler (Agilent Technologies, Palo Alto, CA), a Knauer 120 HPLC pump (Knauer, Berlin, Germany) and a Merck L-7490 refractive index detector (Merck, Darmstadt, Germany). The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm x 0.77 cm i.d., 5 μm particle size) packed with porous, highly cross-linked styrene-divinylbenzene copolymers (Agilent Technologies, Palo Alto, CA) connected in series. Tetrahydrofuran at a flow rate of 1 mL/min was the mobile phase. The relative concentrations of the different components were estimated considering the same response factor for all the analytes.

GC-MS analysis

The TA samples were derivatized by applying a silylation reaction prior to the CG-MS analysis. A volume of 300 μL of the silylation reactant, BSTFA containing 1% of TMCS, was added to an aliquot of 10 mg of TA concentrates. The reaction was performed at 100 °C for 30 min. Then the solution was directly analyzed by GC-MS. A Finnigan Trace-GC 2000 gas chromatograph coupled to a Polaris-Q Ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA) operating in full scan and the MS/MS mode was used. The chromatograph was equipped with a split/splitless injector that operated at 285 °C in the split mode with a 20:1 split ratio. The column used was a Zebron ZB-5ms (Phenomenex, Torrance, CA, USA) fused silica capillary column (30 m long × 0.25 mm i.d × 0.25 μm film thickness). Helium at 1 mL/min at constant flow was the carrier gas. The initial temperature was 150 °C, which was kept for 5 min, then raised to 290 °C at a rate of 5 °C /min and held for 50 min. The electron

146 energy applied was 70 eV and the scan range used was 60-700 amu. The Xcalibur software
147 (version 1.4) was used for data acquisition and processing.

148 **Analyses of antioxidant properties**

149 A Synergy™ HT-multimode microplate reader with an automatic reagent dispenser and a
150 temperature controller from Biotek Instruments (VT, USA) was used in the different
151 antioxidant assays. Biotek Gen5™ data analysis software was used. Each 96-well plate was
152 designed to assay four repetitions per sample and standard, four levels of standards for
153 calibration, and eight repetitions per blank or control. The reactions were initiated by
154 automatic addition of 60 μL of the reactant solutions in the DPPH, ABTS, FRAP and ORAC
155 assays, respectively. In all assays, two different solutions of the TA samples were measured.

156 First, a solution was made by adding to 100 mg of sample 10 mL of an acid ethanolic solution
157 prepared with ethanol, water and 2 M HCl at a relative proportion of 1:1:0.02 (v/v). The
158 solution was shaken in a vortex for 20 min, centrifuged at 5000 rpm for 20 min and the
159 solvent was separated. The procedure was repeated again by adding 4 mL of the ethanolic
160 solution to the precipitate. Both ethanolic phases were joined and measured. The other
161 solution tested was that prepared by dissolving the concentrates in absolute ethanol at a
162 concentration of 0.5 mg mL^{-1}. All samples were completely dissolved at this concentration.
163 The results obtained with the two solutions were not different between each other. Results of
164 the second solution were presented only. Pure maslinic acid and oleanolic acid dissolved at
165 0.5 mg/mL in absolute ethanol were also tested. In addition, BHT and α-tocopherol dissolved
166 in absolute ethanol were also measured as reference antioxidants.

167 DPPH assay

168 The procedure used was that described by Morales *et al.*[18] DPPH absorption decay was
169 recorded at 520 nm for 10 min. The antiradical activity was determined at 10 min and this was

expressed as Trolox equivalent antioxidant capacity (TEAC) using a calibration curve with Trolox solutions in absolute ethanol at concentrations of 40, 100, 200 and 400 μM. All the reaction mixtures were prepared in duplicate and four independent assays were run for each sample. The limit of quantification was 10 μM.

ABTS assay

The free radical scavenging activity was also evaluated against the ABTS radical cation, according to Morales *et al.*[18] with minor modifications. The ABTS radical was measured at 734 nm and data were recorded for 10 min. The antiradical activity was also determined at 10 min and expressed as TEAC using a calibration curve with Trolox dissolved in absolute ethanol. All the reaction mixtures were prepared in duplicate and four independent assays were run for each sample. The limit of quantification was 40 μM.

FRAP assay

The ferric reducing ability was determined according to Morales *et al.*[18] It was based on the reduction reaction of the Fe^{3+}-TPTZ complex to the Fe^{2+} form under acidic conditions, which generates an intense blue color with a maximum light absorption at 595 nm. Only the samples prepared in the acidified ethanolic solution were measured. Similar to the DPPH and ABTS assays, the ferric reducing ability was expressed as TEAC using a calibration curve with Trolox. All the reaction mixtures were prepared in duplicate and four independent assays were run for each sample. The limit of quantification was 10 μM.

ORAC assay

The peroxyl radical scavenging activity was determined by applying the ORAC assay also described by Morales *et al.*[18] Fluorescein oxidation was induced by the water soluble azo initiator APPH at 37 °C. The reaction was carried out in a 75 mM phosphate buffer at pH 7.4. The fluorescence decay of fluorescein was recorded for 90 min using 485 and 528 nm as

194 excitation and emission wavelengths, respectively. The radical scavenging activity was
195 determined using the data recorded at 90 min. All the reaction mixtures were prepared in
196 duplicate, and four independent assays were performed for each sample. The antiradical
197 activity was also expressed as TEAC using a calibration curve with Trolox dissolved in water.
198 The limit of quantitation was 5 μM.

199 **Analysis of phenolic compounds**

200 The analysis of phenolic compounds was made according to Romero *et al.*[19] A volume of 20
201 μL of a solution of the concentrates in ethanol at 20 mg/mL was analyzed directly in the same
202 HPLC chromatograph described above for the analysis of TA. Phenolic compounds were also
203 separated onto the same HPLC column at 35 °C using a gradient elution with water (pH 2.5
204 adjusted with 0.15% phosphoric acid) (A) and methanol (B). The initial composition was 90%
205 A and 10% B. The concentration of B was increased to 30% in 10 min and held for 20 min.
206 Then, B was raised to 40% in a period of 10 min, held for 5 min, and then increased to 50%.
207 Finally, B was increased to 60, 70 and 100% in 5-min periods. The initial conditions were
208 reached in 10 min. Chromatograms were recorded at 280 and 240 nm for phenolic and
209 oleosidic compounds, respectively. Quantification was obtained by external calibration.

210 **Statistical analysis**

211 Unless indicated, analytical determinations were carried out in triplicate and results were
212 expressed as mean values followed by the standard deviation. Comparisons between extracts
213 obtained from oils produced in the same period were made by Student's *t* test in Microsoft
214 Excel 2010 (Microsoft Corporation, Redmond, WA, USA). A multivariate general linear
215 model (GLM) in 24.0 SPSS Statistics program (SPSS Inc., Chicago, IL, USA) was applied to
216 examine possible significant differences between samples extracted from oils obtained by
217 centrifugation and subsequent hexane extraction. The season, harvest period and type of oil

were considered the independent factors, whereas the data obtained in the different antioxidant tests, the total content of phenols and the content of *o*-diphenols were the dependent factors. Due to the fact that the null hypothesis in the variance homogeneity test or Levene's test was not fulfilled, a non-parametric test was also applied in the SPSS program. The U test of Mann-Whitney considering independent samples was the test used. Linear correlation analysis was applied in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) to determine correlations between the antioxidant tests and the contents of phenolic compounds. Significance was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction and characterization of TA concentrates

The extraction yield of TA was much greater from the oils obtained by centrifugation than those obtained by subsequent solvent extraction. It ranged between 2.5 and 48.4 g kg^{-1} oil in the C samples and between 1.2 and 3.0 g kg^{-1} oil in the S samples (**Table 1**). The lower yields found from the oils obtained by solvent extraction were consistent with the lower amounts of TA reported in crude oils.[2] This fact was attributed to two fundamental reasons. First, after the oil extraction by centrifugation, the TA levels in the "alperujo" decreased because a great part was removed along with the oil. Second, the low solubility of TA in hexane also contributed to a lower amount of these compounds in the oils extracted with solvent compared to those obtained previously by centrifugation.[2]

The amount of extract from the C oils increased along the season period in the two seasons studied, whereas no pattern was found for the S samples. This fact was coherent with the increase of TA observed in the oil phase of "alperujo" during its storage time in large ponds in a previous study.[2]

241 The analysis of TA in the concentrates showed the presence of oleanolic and maslinic acids in
242 high amounts (**Table 2**). With the exception of sample 6, the total levels of TA were
243 substantially greater for the C samples. These ranged between 850 and 980 g kg^{-1} for the C
244 samples and between 510 and 900 g kg^{-1} for the S samples. Clear differences were also
245 observed in the TA composition between the C and S samples. While the most abundant TA
246 in the C samples was maslinic acid (MA), showing concentrations that varied between 575
247 and 698 g kg^{-1} sample, oleanolic acid (OA) was predominant in the S samples, with
248 concentrations that ranged between 405 and 703 g kg^{-1} sample. On average, the relative
249 concentrations of OA and MA were, respectively, 30:70 (p/p) for the C samples and 77:23
250 (p/p) for the S samples. Therefore, concentrates rich in MA and OA, respectively, were
251 obtained from the oils extracted by centrifugation and subsequent extraction with hexane. In
252 this respect, results reported for crude olive pomace oils also showed higher amounts of MA
253 than OA in oils obtained by centrifugation and the opposite was true for the oils obtained by
254 subsequent extraction with hexane.[2] However, the relative concentrations of OA and MA
255 were different to those found in the present study for the concentrates. On average, the
256 relative concentrations of OA and MA were reported to be, respectively, 43:45 (p/p) for the
257 oils extracted by centrifugation and 87:13 (p/p) for those obtained by solvent extraction.[2] The
258 higher concentration of OA in the oils extracted with solvent was mainly attributed to its
259 greater solubility in hexane than MA. Similarly, the great solubility differences in hexane
260 between OA and MA could also explain the much higher relative concentration of MA found
261 for the C samples in the present study compared to the values reported in oils.[2] In this regard,
262 substantial amounts of OA could have been removed in the washing step with hexane before
263 the extraction of TA with ethanol.

264 An HPSEC analysis was applied first to gain an insight about what type of compounds other
265 than TA were present in the concentrates. This is the analysis used in the evaluation by

molecular weight of the groups of compounds that form the polar fraction of used frying oils.[20] HPSEC chromatograms showed the presence of tri-, di- and mono-acylglycerols, apart from TA, which eluted as a single chromatographic peak (not shown). The sum of tri-, di- and mono-acylglycerols was used as an estimate of the amount of residual oil in the concentrates (**Table 3**). Samples 1S and 6C showed amounts of oil as high as 240 and 108 g kg^{-1} sample, respectively, which indicated an inefficient wash with hexane. The amount of residual oil varied between 10 and 70 g kg^{-1} for the rest of samples. The results for samples 1S and 6C were coherent with their relatively low values of TA (Table 2). However, the amount of residual oil did not explain the relatively low values of TA in a few samples, such as sample 4S, which presented an oil content of 10 g kg^{-1} sample only. This fact suggested that the concentrates also comprised compounds that eluted along with the TA in the HPSEC analysis.

A GC-MS analysis of the concentrate samples derivatized with TMSi exhibited the presence of free fatty acids and confirmed the occurrence of monoacylglycerols (not shown). The fatty acids detected were palmitic, oleic, linoleic and linolenic acids. The free fatty acids were therefore compounds that eluted along with the TA in the HPSEC analysis. Although not observed in the GC-MS analysis, other compounds that could be present in the concentrates of TA because of their polarity could be aliphatic alcohols. These form part of the minor fraction in crude olive pomace oils and are present at substantial amounts.[2] In a previous study, the concentrations of aliphatic alcohols detected in crude olive pomace oils obtained by hexane extraction were substantially greater than those determined in their corresponding oils produced by previous centrifugation.[2] If present, similarly to free fatty acids, aliphatic alcohols would also elute along with TA in the HPSEC analysis and also would contribute to the less purity found for the S samples.

Evaluation of the potential antioxidant activity of TA concentrates

All concentrates showed antiradical activity towards DPPH and ABTS (**Figure 1A**). Compared to chain-breaking antioxidants like α-tocopherol and BHT, the antiradical activities of the concentrates found in the two tests were two orders of magnitude lower (data not shown). The activity values in the DPPH test were similar for each pair of C and S samples, although slight significant differences were detected when analyzing the mean values individually. A global analysis of all samples did not show a definite pattern. In fact, a non-parametric test did not show significant differences between the C and S samples. Similar results were also found in the ABTS test.

Despite the antiradical activity found in the concentrates, pure OA and pure MA did not display activity in the DPPH test at the same experimental conditions. MA did not either show antiradical activity towards ABTS, whereas OA exhibited certain activity that was low (10 μmol/g TEAC) but comparable to the concentrates that showed the lowest values (Figure 1A).

The DPPH and ABTS tests also provided values with a decreasing trend as the season progressed in each harvest. In addition, an increased antiradical activity was also observed in the second season compared to the first one.

The TA concentrates also showed ferric reducing power and the results obtained were not different to those of the antiradical activity tests (**Figure 1B**). The TEAC values were two orders of magnitude lower than those found for α-tocopherol and BHT. While the S samples in the first season displayed significantly greater reducing power than their C sample homologs, a definite pattern was not found in the following harvest. With the exception of the S samples in the first harvest, it was also observed that the reducing power decreased as the season progressed. Also, the concentrates obtained from the oils of the second harvest generally showed on average greater values. Pure MA and OA did not either present ferric reducing power.

The TA concentrates also presented antiradical activity in the ORAC assay (**Figure 1C**). Although significant differences were found between the S and C samples obtained from oils of a given period, a definite pattern along each season was not observed. A clear trend of activity losses along a harvest was not found, but greater activity on average was detected in the second harvest compared to the previous one. Pure MA and OA displayed very slight antioxidant activity, which was from one to two orders of magnitude less than the activities found for the TA concentrates.

The results obtained in the different tests for pure OA agreed with those reported by Castellano *et al.*[17] These authors found that OA showed no activity in inhibiting the DPPH radical, a low activity in scavenging the ABTS radical, and very low activity in protecting fluorescein in the ORAC assay. In contrast, Allouche *et al.*[14] did observe radical scavenging activities for both OA and MA in the DPPH and ABTS tests, but these were very weak and dependent on the TA concentrations. In fact, a high DPPH scavenging activity was found for MA when its concentration was 5 mol per mol of DPPH or higher. Therefore, the results of the antioxidant tests seem to be dependent on the experimental conditions and, for this reason, discrepancies between different studies could be justified.[21] The antioxidant role attributed to TA in numerous pathological processes goes, however, beyond the very weak antiradical activity observed through tests that are even subjected to experimental conditions. As an example, OA has been reported to exert an antioxidant effect through indirect biological actions, as it was able to induce the expression of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase.[22-26]

Given that the concentrates and the pure TA were assayed at the same experimental conditions, the results obtained in the antioxidant tests suggested that the slight antioxidant activity found in the concentrates were not due to the TA. Other compounds could have been involved instead. In this regard, phenolic compounds of strong antioxidant activities are

known to be present in virgin olive oils.[27] For this reason, the possible presence of phenolic antioxidants in the TA concentrates was investigated. Preliminary assays gave positive results in the Folin-Ciocalteu test (not shown),[28] indicating the occurrence of phenolic compounds in the TA concentrates. Thus, a detailed analysis of phenolic compounds was carried out for characterization purposes. Results showed the presence of phenolic antioxidants at a concentration range of g kg^{-1} sample in all concentrates (**Table 4**). Samples C presented greater levels of *p*-Coumaric acid, Luteolin and Apigenin and less contents of Hydroxytyrosol-1-glucose and Vanillic acid than their S sample homologs. The levels of Hydroxytyrosol were variable, being higher in the C samples than in their S sample counterparts in the first harvest and lower in the following one. Tyrosol however showed a definite trend to be more concentrated in the S samples.

The total levels of phenolic compounds were examined to determine whether the results obtained in the antioxidant tests were mainly due to the action of phenolic antioxidants. The total levels of phenols were similar between the C and their S sample homologs in the first season, although statistical different at the beginning and the end of the harvest. In contrast, remarkable differences were found in the second harvest. The S samples showed quite higher total levels of phenolic compounds. These results were consistent with the levels of phenolic compounds found for crude olive pomace oils in a previous report.[2] Those extracted with hexane presented significantly higher contents than those obtained by centrifugation. The similar levels of phenolic compounds found for the C and their S sample counterparts in the first season were consistent with the similar activities found in the antioxidant tests and with the fact that definite patterns for the C and S samples were not found. In contrast, the higher levels found for the S samples in the second season did not give rise to definite antioxidant patterns for the C and S sample homologs. Nevertheless, the total levels of phenols were

363 coherent with the antioxidant activity losses observed along each season and with the greater
364 antioxidant activities found in the second harvest (Table 4).

365 The decrease of phenolic compounds found in the concentrates along each harvest may be
366 related to the decreased contents in the fruit during ripening.[29] In addition, the lower levels of
367 phenols found in the concentrates of the first harvest could be associated to losses during the
368 storage of the crude oils before being filtered. Unlike the oils of the second harvest, which
369 were used immediately as they were received, the oils of the first season had been stored at
370 refrigeration conditions for one year when they were filtered.

371 It is known that the phenolic compounds of greater antioxidant activity are those with two
372 hydroxyl groups in *ortho*- position, the so called *ortho*-diphenols.[30] The levels of *o*-diphenols
373 were also examined to try to relate them with the results obtained in the antioxidant tests. The
374 *o*-diphenols were formed by Hydroxytyrosol and its derivatives, and Luteolin. Their contents
375 followed a similar trend to that found for the total levels of phenolic compounds, except that
376 the C samples of the first harvest showed significantly greater contents than their S sample
377 counterparts (not shown). Therefore, the contents of *o*-diphenols did not provide additional or
378 complementary information to the total levels of phenolic compounds.

379 A statistical analysis of linear correlation showed high, positive correlation coefficients
380 between the total amount of phenolic compounds and the DPPH and ABTS tests, respectively
381 (**Table 5**). The linear relationship found for the total phenols and the FRAP was moderate,
382 whereas that with the ORAC assay was weak.

383 **CONCLUSIONS**

384 Results have shown that concentrates of TA, containing MA and OA, can be readily obtained
385 from a byproduct generated by filtration of crude olive pomace oils extracted by
386 centrifugation or with hexane. Concentrates isolated from oils obtained by centrifugation were

rich in MA, whereas those from oils extracted with hexane were rich in OA. The concentrates showed slight antioxidant properties that can be mainly attributed to the presence of phenolic compounds at levels of g kg^{-1} sample.

ACKNOWLEDGEMENTS

This work was funded by the Spanish Ministry of Economy, Industry and Competitiveness through project AGL2013-45110-R. We would also like to thank "Oleícola El Tejar" for providing the oils, Irene Pérez de la Rosa for technical assistance, Concepción Romero Barranco for the HPLC analyses of TA and phenolic compounds and José Julián Ríos Martín for the GC-MS analysis.

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Figure captions

Figure 1. Evaluation of antioxidant activity of TA concentrates expressed as Trolox Equivalent Antioxidant Capacity (TEAC). For sample names see Table 1. Different letters for a given pair of C and S samples obtained from oils produced in a given season period denote significant differences ($p<0.05$).

Table 1 Results of oil filtration and extraction of triterpenic acids (TA).

Samples	Oil (g)	Oil retained (g)	Extract 1 (g)	Extract 2 (g)	Extract 2 (g kg^{-1} oil)
1C	874.0	58.1	57.4	2.2	2.5
1S	829.0	137.7	135.6	1.8	2.2
2C	824.8	141.0	139.1	4.4	5.3
2S	787.7	227.6	226.1	2.2	2.8
3C	853.3	249.4	209.7	33.6	39.4
3S	859.1	174.3	175.2	1.3	1.5
4C	797.7	179.1	174.0	5.1	6.4
4S	831.7	183.4	183.2	2.5	3.0
5C	793.2	217.2	209.2	11.8	14.9
5S	819.8	180.8	179.8	1.0	1.2
6C	807.7	262.7	230.5	39.1	48.4
6S	818.2	132.2	131.1	1.8	2.2

1, January 13; **2**, April 13; **3**, May 13; **4**, December 13; **5**, March 14; **6**, June 14; **C**, extract obtained from the oil produced by Centrifugation; **S**, extract obtained from the oil produced by subsequent Solvent extraction; **Oil retained**, oil retained in the paper filter after filtration; **Extract 1**, oil retrieved in the washing step with hexane; **Extract 2**, concentrate of TA obtained by subsequent extraction with ethanol.

Table 2 Quantitative analysis of triterpenic acids in the TA concentrates.

Samples	OA (g kg^{-1} sample)	MA (g kg^{-1} sample)	Total (g kg^{-1} sample)
1C	316 ± 14	575 ± 19	891 ± 33
1S	451 ± 11	98 ± 1	549 ± 13
2C	296 ± 25	678 ± 7	974 ± 23
2S	548 ± 35	191 ± 9	738 ± 35
3C	292 ± 7	687 ± 7	980 ± 12
3S	544 ± 12	209 ± 8	753 ± 20
4C	273 ± 3	646 ± 7	919 ± 10
4S	405 ± 30	110 ± 3	515 ± 31
5C	250 ± 4	698 ± 18	948 ± 21
5S	703 ± 19	152 ± 5	855 ± 23
6C	231 ± 4	624 ± 2	856 ± 5
6S	632 ± 9	268 ± 5	900 ± 14

OA, Oleanolic acid; **MA**, Maslinic acid. See Table 1 for other abbreviations. Results are expressed as mean values followed by the standard deviation of three analytical determinations.

Table 3 HPSEC analysis of the TA concentrates.

Samples	TAG (g kg^{-1} sample)	DAG (g kg^{-1} sample)	MAG (g kg^{-1} sample)	FFA* (g kg^{-1} sample)	Oil (g kg^{-1} sample)
1C	23	22	7	948	52
1S	193	31	16	760	240
2C	5	19	5	971	29
2S	22	10	7	961	39
3C	12	18	3	967	33
3S	10	17	6	967	33
4C	31	22	5	942	58
4S	10	nd	nd	990	10
5C	29	15	4	952	48
5S	35	17	nd	948	52
6C	77	27	4	892	108
6S	53	13	4	930	70

TAG, Triacylglycerols; **DAG**, Diacylglycerols; **MAG**, monoacylglycerols; **FFA***, group of compounds that normally comprises free fatty acids and other oil minor components in oil HPSEC analyses but it is mainly constituted by triterpenic acids in the analysis of the TA concentrates studied; **Oil**, obtained from the sum of TAG, DAG and MAG; nd, not detected. See Table 1 for other abbreviations. Results express mean values of two analytical determinations.

Table 4 Analysis of phenolic compounds (g kg^{-1} sample) in the TA concentrates.

Samples	Hy	Hy1G	Sa	Ty	VA	*p*-CoA	Lut	Apig	Others*	Total
1C	3.42 ±0.13	0.064 ±0.000	0.037 ±0.002	0.59 ±0.01	0.037 ±0.000	0.136 ±0.010	0.229 ±0.007	0.076 ±0.000	0.047 ±0.002	4.63 ±0.16
1S	3.00 ±0.15	0.150 ±0.005	0.081 ±0.005	0.73 ±0.04	0.087 ±0.005	0.015 ±0.001	0.032 ±0.004	nd	nd	4.10 ±0.20
2C	1.79 ±1.12	0.033 ±0.003	nd	0.41 ±0.01	0.029 ±0.002	0.061 ±0.002	0.232 ±0.017	0.070 ±0.004	0.010 ±0.01	2.64 ±0.16
2S	0.26 ±0.03	0.048 ±0.001	nd	2.28 ±0.14	0.178 ±0.015	0.015 ±0.000	0.012 ±0.002	nd	nd	2.80 ±0.19
3C	0.94 ±0.02	nd	nd	0.23 ±0.03	nd	0.051 ±0.006	0.159 ±0.013	0.026 ±0.001	nd	1.41 ±0.04
3S	nd	nd	nd	1.87 ±0.12	0.220 ±0.031	nd	nd	nd	nd	2.09 ±0.16
4C	4.04 ±0.60	1.077 ±0.312	0.106 ±0.027	0.78 ±0.07	0.044 ±0.004	0.052 ±0.011	0.262 ±0.067	0.023 ±0.004	0.31 ±0.05	6.70 ±1.16
4S	4.50 ±0.01	4.733 ±0.020	0.611 ±0.009	0.64 ±0.03	0.043 ±0.004	0.012 ±0.001	nd	nd	0.74 ±0.11	11.27 ±0.09
5C	0.27 ±0.03	nd	nd	0.66 ±0.05	0.045 ±0.007	0.054 ±0.009	0.206 ±0.031	0.031 ±0.027	nd	1.27 ±0.06
5S	3.72 ±0.04	1.154 ±0.064	0.089 ±0.021	1.92 ±0.05	0.191 ±0.004	nd	nd	nd	0.13 ±0.01	7.21 ±0.09
6C	0.89 ±0.08	nd	nd	0.41 ±0.02	0.032 ±0.004	nd	0.061 ±0.053	0.017 ±0.015	nd	1.41 ±0.13
6S	0.91 ±0.13	nd	nd	1.09 ±0.10	0.088 ±0.004	nd	nd	nd	nd	2.09 ±0.13

Hy, Hydroxytyrosol; **Hy1G**, Hydroxytyrosol-1'-*β*-glucoside; **Sa**,Salidroside; **Ty**, Tyrosol; **VA**, Vanillic acid; ***p*-CoA**, *p*-Coumaric acid; **Lut**, Luteolin; **Apig**, Apigenin; nd, not detected. See Table 1 for other abbreviations.*Hydroxytyrosol glycol, Hydroxytyrosol acetate, Caffeic acid and Comselogoside. Results are expressed as mean values followed by the standard deviation of three analytical determinations.

o-diphenols: Hydroxytyrosol, Hydroxytyrosol-1-glucose, Hydroxytyrosolglycol, Hydroxytyrosol acetate and Luteolin

Table 5 Correlation coefficients found between the methods of antioxidant activity and the amounts of *ortho*-diphenols and total phenols.

	***ortho*-diphenols**	**Total phenols**	**DPPH**	**ABTS**	**FRAP**	**ORAC**
***ortho*-diphenols**	1					
Total phenols	0.9727	1				
DPPH	0.7450	0.6857	1			
ABTS	0.7893	0.8052	0.6968	1		
FRAP	0.5773	0.6462	0.5611	0.8547	1	
ORAC	0.3377	0.3345	0.4708	0.3113	0.4267	1